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Molecular cloning, expression, and characterization of E2F transcription factor 4 from *Antheraea pernyi*

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Abstract

The E2F transcription factor family is distributed widely in eukaryotes and has been well studied among mammals. In the present study, the E2F transcription factor 4 (E2F4) gene was isolated from fat bodies of *Antheraea pernyi* and sequenced. *E2F4* comprised a 795 bp open reading frame encoding a deduced amino acid sequence of 264 amino acid residues. The recombinant protein was expressed in *Escherichia coli* (Transetta DE3), and anti-E2F4 antibodies were prepared. The deduced amino acid sequence displayed significant homology to an E2F4-like protein from *Bombyx mori* L. Quantitative real-time polymerase chain reaction analysis revealed that *E2F4* expression was highest in the integument, followed by the fat body, silk glands, and haemocytes. The expression of *E2F4* was upregulated in larvae challenged by bacterial (*Escherichia coli*, *Micrococcus luteus*), viral (nuclear polyhedrosis virus), and fungal (*Beauveria bassiana*) pathogens. These observations indicated that E2F4 is an inducible protein in the immune response of *A. pernyi* and probably in other insects.

Keywords: quantitative real-time PCR, silkworm, genome, homology

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Introduction

The E2F transcription factor family is distributed widely in eukaryotes. Currently, this family comprises eight genes (*E2F1*, *E2F2*, *E2F3a*, *E2F3b*, *E2F4*, *E2F5*, *E2F6*, *E2F7*, and *E2F8*) (Attwooll *et al*., 2004; Iaquinta & Lees, 2007); however, this number might increase as new members are discovered in mammals and homologs are identified in other eukaryotes (Dyson, 1998; Nevins, 1998). On the basis of their structures and physiological functions, the E2F family members are classified into transcriptional activators (E2F1 to E2F3a) and transcriptional repressors (E2F3b to E2F8) (Iaquinta & Lees, 2007). They function in cell cycle progression and differentiation, as regulators of apoptosis, and in tumour suppression. The application of new technologies has expanded the number and nature of genes regulated by E2F. The functions of these genes range from cell growth regulators to genes involved in differentiation, development, DNA repair, recombination and apoptosis, mRNA processing, and includes genes with no identified function (DeGregori, 2002; Cam & Dynlacht, 2003).

The Chinese oak silkworm (*Antheraea pernyi* (Guerin-Menebille)) is a wild insect species whose larvae feed on oak plant leaves. They are reared in several Asian countries, such as China, Korea, and India. The *A. pernyi* larvae are important producers of raw silk, and in China alone almost 70,000,000 kg of cocoons (pupae) are produced each year, which contribute 90% of the world's production (Chang *et al*., 2003; Wei *et al*., 2008; Liu *et al*., 2010). Furthermore the larvae, pupae, and moths are edible and contain high quality proteins, which constitute all the amino acids required for human nutrition. They are also used to produce cosmetics. Their pupae contain 45–55% protein as dry matter, which could raise haemoglobin and serum total protein levels significantly when fed to rats, producing protective effects on the liver in a carbon tetrachloride-induced rat hepatic injury model (Yang *et al*., 2002; Zhou & Han, 2006).

In the present study, we investigated E2F transcription factor 4 (E2F4) an important member of the E2F family in *A. pernyi* (Saturniidae: Lepidoptera). The *E2F4* gene has been studied extensively in animals, particularly in mammals, and in plants (Müller & Helin, 2000; Lammens *et al*., 2009); however, there is a lack of literature on E2F4 in insects.

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Table 1. Primers used in the present study.

Primer	Sequence (5′–3′)	Tm (°C)	Purpose
F E2F4	CGGGATCCACTGATCCAAAGTAT	55	Expression
R E2F4	CCGCTCGAGACAAGGTATATCAAAC	55	Expression
18SF	CGATCCGCCGACGTTACTACA	55	qRT-PCR
18SR	GTCCGGGCCTGGTGAGATTT	55	qRT-PCR
RT-F E2F4	GCAGGAAGCGAAAGATGGAG	55	qRT-PCR
RT-R E2F4	CCAGCCCTCTCCATTGTAT	55	qRT-PCR

Therefore, the present study aimed to characterize E2F4 in *A. pernyi*. We cloned the *E2F4* gene and described its expression profile following challenge with viral, bacterial, and fungal pathogens. Moreover, we determined the expression of *E2F4* in different developmental stages of *A. pernyi*. This basic knowledge will provide the foundation for further studies to explore the immune functions of E2F4.

Materials and methods

Experimental organism

The present study was conducted in the Silkworm Immunology laboratory, Department of Biochemistry and Molecular Biology, Anhui Agricultural University, Hefei, China. Pupae of *A. pernyi* were obtained from the Sericultural Research Institute of Liaoning, China, and raised to adults. Eggs were collected and incubated until hatching. The larvae were fed on fresh oak leaves and kept at room temperature, with a 10:14-h light:dark photoperiod, and 70% relative humidity until they transformed into pupae.

RNA extraction and cloning of E2F4 transcription factor

Total RNA was extracted from the fat bodies using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was synthesized using TransScript Synthesis SuperMix (TransGen, Beijing, China). Oligonucleotide primers were designed using the Primer Premier 5.0 software package to amplify a fragment of *E2F4* (table 1). Polymerase chain Reaction (PCR) was performed using the following amplification program: 5 min at 94°C; followed by 35 cycles at 94°C for 1 min, 55°C for 40 s and 72°C for 1 min; and a final elongation step at 72°C for 10 min. The PCR product was resolved by 1% agarose gel electrophoresis and analysed. Invitrogen than sequenced the obtained product.

Nucleotide sequence analysis

NCBI bioinformatics tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to determine conserved domains in the predicted E2F4 protein. Furthermore, the molecular weight of the E2F4 was calculated by ExPASy (http://web.expasy.org/compute_pi/). Multiple sequence alignments were performed using the ClustalX package with its default parameters (Livak & Schmittgen, 2001). A phylogenetic tree was constructed with MEGA 5.1 using the neighbour-joining algorithm method (Tamura *et al*., 2011), with a bootstrap test of 1000 replications.

Prokaryotic expression and protein purification

A pair of specific primers (F E2F4 and R E2F4) was designed to amplify the 803 bp DNA fragment comprising the entire open reading frame (ORF) of *E2F4*. The ORF was cloned into cloning vector pMD-19T, which was then digested with Bam HI and Xho I, and the fragment containing the ORF was ligated into the pET-30a(+) vector (Novagen, USA). The insertion of the correct fragment was confirmed in the recombinant plasmid, named pET-30a-E2F4, by DNA sequencing, and transformed into *Escherichia coli* Transetta (DE3) cells (Novagen, USA) for protein expression. The Transetta (DE3) cells were cultured in Luria-Bertani media for 4 h and then subjected to isopropyl-β-D-thiogalactopyranoside (IPTG) induction at a final concentration of 0.8 mM in the culture medium. After 12 h of culture, the cells were harvested by centrifugation at 8000 ***g*** for 5 min. The pellets (cells) were suspended in binding buffer (20 mM Tris–HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9) and disrupted by sonication on ice. The disrupted cells were centrifuged at 12,000 ***g*** for 20 min at 4°C, and recombinant protein was purified using the QIAexpress® Ni-NTA Fast Start Kit (Qiagen, Germany), according to the manufacturer's protocol. The recombinant protein was analysed by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using antibodies prepared as detailed in the next section.

Antibody preparation

The antiserum was prepared according to a previously described method (Harlow & Lane, 1999). New Zealand white rabbits were immunized three times for 2-weeks with 100 µg eluted E2F4 protein homogenized in complete Freund's adjuvant. A booster injection was administered 1 week later. The rabbit serum was collected 7 days after the final immunization and stored at −80°C. A monoclonal anti-6-His antibody (Qiagen, Germany) was used to confirm protein expression and the molecular weight.

Expression analysis using quantitative real-time PCR (qRT-PCR)

The total RNA was extracted from fat bodies, haemocytes, midguts, silk glands, Malpighian tubules, and the epidermis of larvae using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The extracted RNA was reverse transcribed into cDNA. Oligonucleotide primers specific for the *E2F4* sequence and the endogenous control (18S rRNA, accession number: DQ347469) were designed using Primer 5.0, based on known sequences (table 1). Real-time PCR was performed in 25 µl reactions containing 12.5 µl of 2× SYBR Premix Ex TaqII (Takara), 1 µl each of the forward and reverse primers, 2 µl of the cDNA, and 8.5 µl of RNase-free H_2O. The

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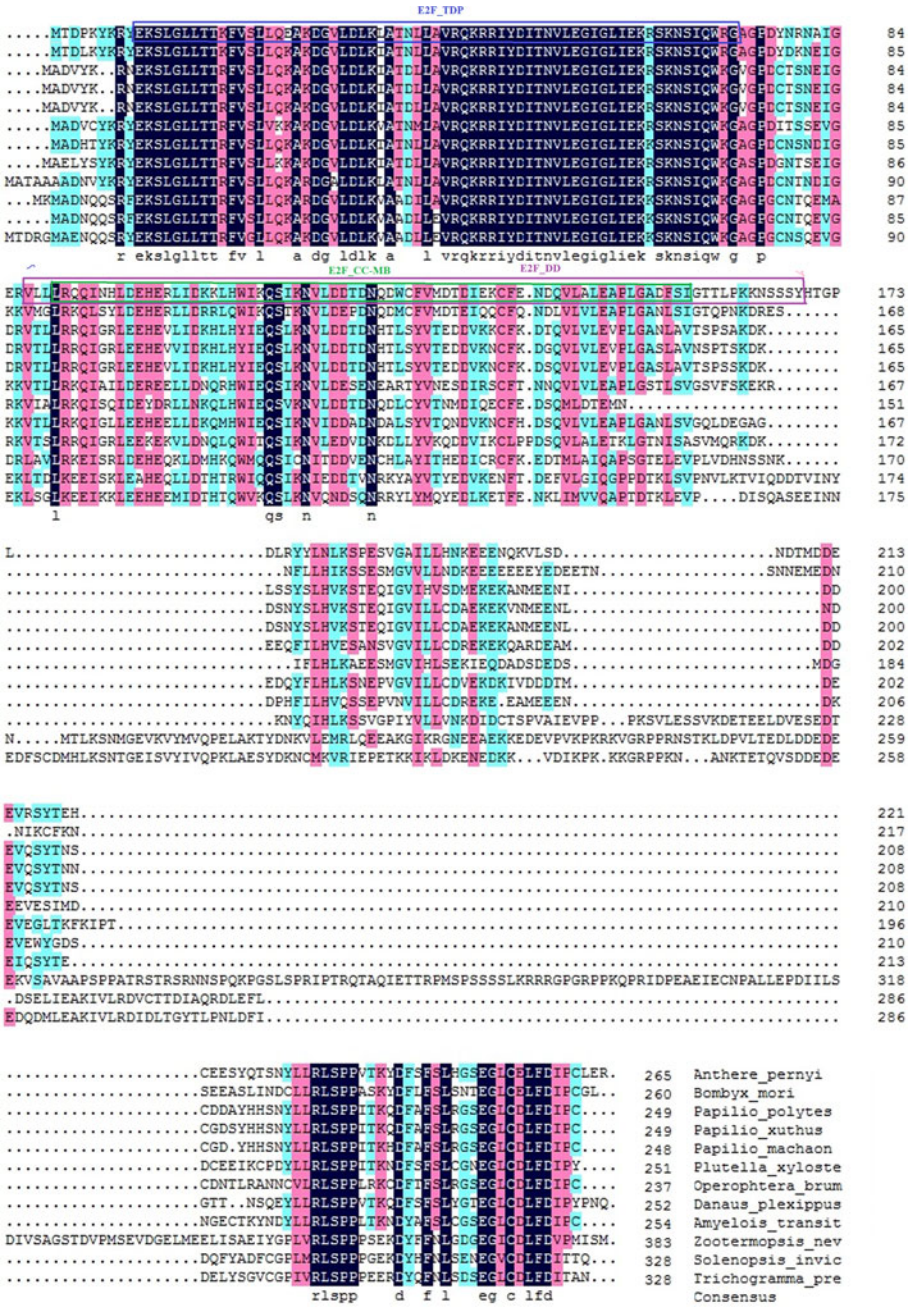

Fig. 1. Alignment of the E2F transcription factor 4 protein with its homologous proteins. The deduced amino acid sequence of E2F transcription factor 4 was aligned with the E2F transcription 4 proteins from *Papilio polytes* (XP_013146090), *Papilio xuthus* (XP_013182302), *Plutella xylostella* (XP_011561624), *Papilio machaon* (KPJ07879), *Zootermopsis nevadensis* (KDR18049), and the E2F transcription factor 4-like protein of *Bombyx mori* (NP_001040298), *Papilio machaon* (XP_014368162), *Operophtera brumata* (KOB76568), *Danaus plexippus* (EHJ67877), *Amyelois transitella* (XP_013190081), *Solenopsis invicta* (XP_011157894), and *Trichogramma pretiosum* (XP_014224938).

amplification program was performed as follows: 95°C for 30 s, followed by 39 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. A melting curve was produced by monitoring the fluorescence continuously while slowly heating the sample from 65 to 95°C. The relative expression level of the *E2F4* gene was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). All qRT-PCR experiments were repeated five times. The expression of the *E2F4* gene in the epidermis was arbitrarily set to 1 and used for normalization.

SDS-PAGE and Western blotting

The different tissues, such as the fat bodies, haemocytes, and mid-gut, were ground in liquid nitrogen and dissolved

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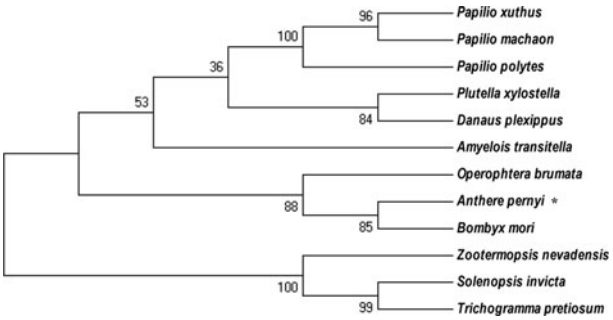

Fig. 2. Phylogenetic analysis of the *A. pernyi* Ap-E2F transcription factor 4 with other E2F4 E2F4-like protein from other species. The deduced amino acid sequences were aligned and a phylogenetic tree was constructed using MEGA (version 5.05) and the neighbour-joining method.

in Radioimmunoprecipitation assay (RIPA) lysis buffer (Sangon, Shanghai, China). The concentrations of the extracted proteins were determined using the bicinchoninic acid (BCA) method. The proteins were subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Sigma, St. Louis, MO, USA) by electrophoretic transfer. Anti-beta-Actin antibodies (Transgen biotech, Beijing, China) were used as loading control primary antibodies. The membranes were blocked with 5% non-fat milk in PBST (PBS containing 0.1% Tween-20) overnight at 4°C, and then washed three times with PBST for 10 min each. The membranes were incubated with the anti-E2F4 antibodies (diluted 1:500 with 5% non-fat milk in PBST) for 2 h at room temperature, washed with PBST, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (TransGen, Beijing, China; diluted 1:2000 with 5% non-fat milk in PBST) for 1 h at room temperature. Immunoreactive protein bands were detected using an HRP-diaminobenzidine (DAB) Detection Kit (Sangon, Shanghai, China).

Immune challenge assays

Fifth-instar larvae of *A. pernyi* were divided into five groups containing three larvae each. The larvae were injected in their abdomen with 5 µl of heat-treated *E. coli* (Migula) (DH5α, 1×10^6 cells), *A. pernyi* nucleopolyhedrovirus (Ap-NPV, 1×10^6 particles), *Micrococcus luteus* (Schroeter) (1×10^6 cells), *Beauveria bassiana* (Bals-Criv) (1×10^6 spores), or phosphate-buffered saline (PBS) as a control. The injections were performed using microliter syringes (Gaoge, Beijing, China), and the injection sites were sealed with Vaseline immediately after injection. The haemolymph and fat bodies were collected at 1.5, 3, 6, 12, 24, and 48 h post-injection. Three larvae were collected as one sample, and the biological sampling protocol was repeated three times. The transcript and protein expression analysis of E2F4 were performed using qRT-PCR or Western blotting analysis, respectively, as described above.

Results

Bioinformatic analysis of the E2F transcription factor 4 cDNA

The isolated 967 bp cDNA fragment of *E2F4* contained a 5′-untranslated region (UTR) of 64 bp, a 3′ UTR of 108 bp

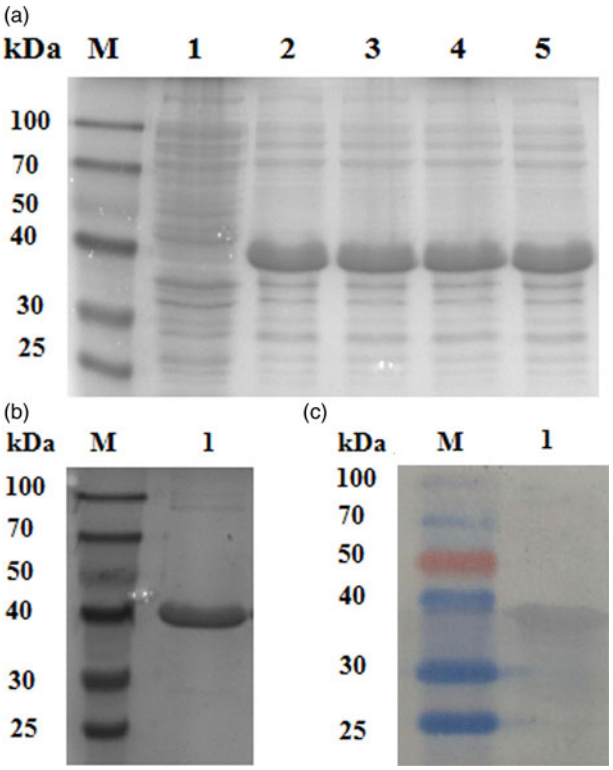

Fig. 3. Protein expression, antibody preparation, and Western blotting analysis. (a) SDS-PAGE of the E2F transcription factor 4 protein expressed in *E. coli* and induced by different isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations. Lane 1: M: molecular marker; lane 2: non-induced expression; lanes 3, 4, 5, and 6: IPTG/Transetta DE3. (b) SDS-PAGE of expressed and purified E2F4 protein. (c) Western blotting analysis of the recombinant E2F4 protein visualized using with anti-His tag antibodies.

and an ORF of 795 bp, encoding a 264 amino acid residue protein, is provided in supplementary figure S1. No signal peptide was found in the deduced protein sequence. The deduced protein had a predicted molecular weight of 30.55 kDa and an isoelectric point of 5.27. BLAST analysis of the whole protein sequence revealed that E2F4 of *A. pernyi* shared high similarity to other insect E2F4 proteins, in particular to the *Bombyx mori* L (fig. 1). E2F transcription factor 4-like protein. The winged-helix DNA-binding (E2F_TDP), E2F_coiled coil-marked box (E2F_CC-MB), and E2F_Dimerization domain (E2F_DD) Putative conserved protein domains were detected in E2F4, is provided in supplementary figure S2. The E2F4 belongs to E2F and DP family. It has ability to bind DNA either as homodimer or as heterodimer in association with TDP1/2; however, the heterodimer increase its binding efficiency. The E2F_CC-MB forms a heterodimer with the corresponding domain of the DP transcription factor that binds the C-terminus of the retinoblastoma (Rb) protein. Furthermore, many authors reported that members containing the third domain (E2F_DD) are involved in several biological processes, such as DNA synthesis, cell cycle progression, proliferation, and apoptosis (Zheng *et al.*, 1999; Rubin *et al.*, 2005; Korenjak *et al.*, 2012). The phylogenetic analysis revealed that E2F4 is phylogenetically close to members from the Lepidoptera, but is more distantly related to the Isoptera and Hymenoptera (fig. 2).

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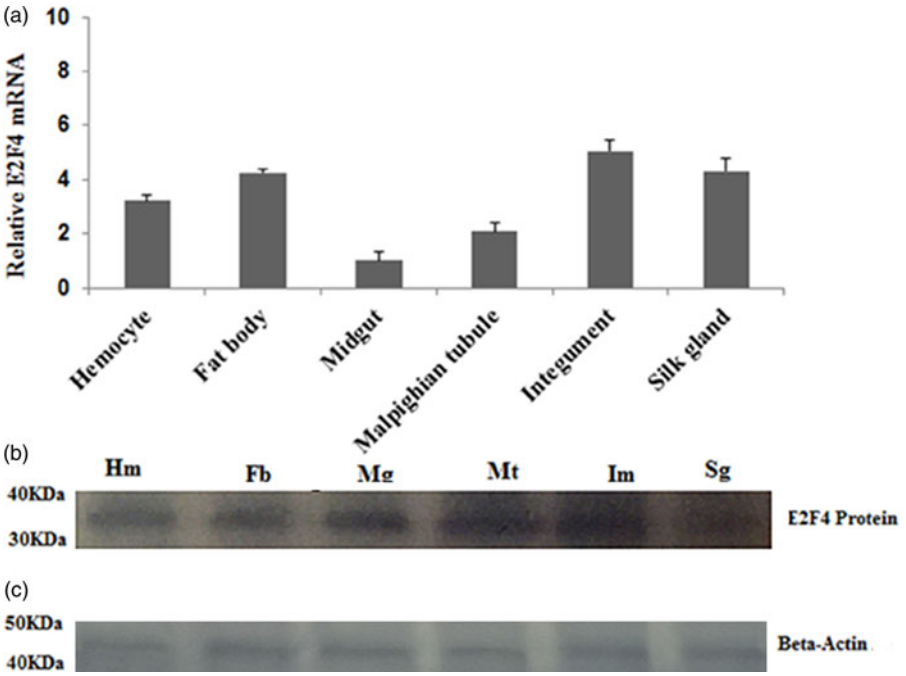

Fig. 4. Expression profile of the E2F4 protein in six different tissues of fifth-instar *A. pernyi* larvae. (a) Real-time PCR analysis of *E2F4* mRNA in larval tissues. (b) Relative expression of E2F4 proteins in fifth-instar *A. pernyi* larval tissues, as analysed by Western blotting using antibodies specific to E2F4. (c) Beta-Actin antibodies were used as a loading control. Hm: Haemocyte; Fb: Fat body; Mg: Mid gut; Mt: Malpighian tubule; Im: Integument; Sg: Silk gland.

Protein expression, antibody preparation, and Western blot analysis

A protein of approximately 30 kDa was observed in protein extracts from *E. coli* induced by IPTG subjected to SDS-PAGE, which was consistent with the predicted size of E2F4 (fig. 3a). Different concentrations of IPTG did not influence the level of recombinant protein expression. A purified E2F × His fusion protein was obtained by affinity chromatography and a protein band corresponding to the predicted molecular weight of 30 kDa was observed (fig. 3b). Western blotting analysis of the recombinant proteins using an anti-His-tag antibody confirmed a consensus protein of 30 kDa (fig. 3c). The purified recombinant protein was then used to prepare rabbit anti-E2F4 antibodies, whose titre was approximately 1:10,000, as determined by an enzyme linked immunosorbent assay (ELISA).

Expression of E2F transcription factor 4 in larval tissues

The *E2F4* mRNA expression level in fat bodies, haemocytes, midguts, epidermis, silk glands, and Malpighian tubules was determined by qRT-PCR. *E2F4* was expressed in all examined tissues; however, its levels in the fat bodies, haemocyte, and integument were higher compared with that in other tissues (fig. 4a). Western blotting analysis of proteins extracted from these tissues produced similar results to the qRT-PCR assays (fig. 4b).

E2F4 Expression profile under pathogenic stress conditions

To understand whether E2F4 is involved in or influences the immune process of *A. pernyi* under biotic stress, we injected bacterial, fungal, and viral pathogens into fifth instar larvae. Total RNA were extracted from the fat body and haemocytes at different time intervals from 1.5 to 48 h, and were then investigated by real-time PCR. When *A. pernyi* larvae were treated with the pathogens, the abundance of E2F4 in the fat bodies and haemocyte varied considerably, the tissue expression varied with the type of pathogen. The viral (nuclear polyhedrosis virus), and fungal (*Beauveria bassiana*) pathogens induced E2F4 expression highly; however the expression level and time of maximum expression varied among them. In fat bodies, NPV produced two expression peaks at 3 and 6 h (fig. 5a), and the maximum expression in response to *B. bassiana* was recorded after 12 h of treatment of (fig. 6a). Meanwhile, two expression peaks were observed (at 24 and 48 h) after *E. coli* challenge (fig. 7a) and three were observed (6, 12, and 48 h) after *M. luteus* challenge (fig. 8a). Approximately similar trends were observed in haemocytes after treatment with the pathogens. The expression fluctuated greatly with the different pathogens and at different time intervals in the haemocytes compared with the fat bodies. NPV showed maximum expression level at 6 h (fig. 5b), whereas *B. bassiana* produced its maximum response at 5 h. The expression level of E2F4 was much higher in response to *E. coli* and *B. bassiana*: however, the maximum level attained by *E. coli* occurred at 3 h, while *B. bassiana* produced its maximum response at 24 h (figs 6b and 7b).

Developmental profile of E2F4 expression

To determine the expression of the E2F4 gene in different developmental stages of *A. pernyi*, we extracted total RNA from egg, larvae (1st to 5th instar), pupae and moth stages

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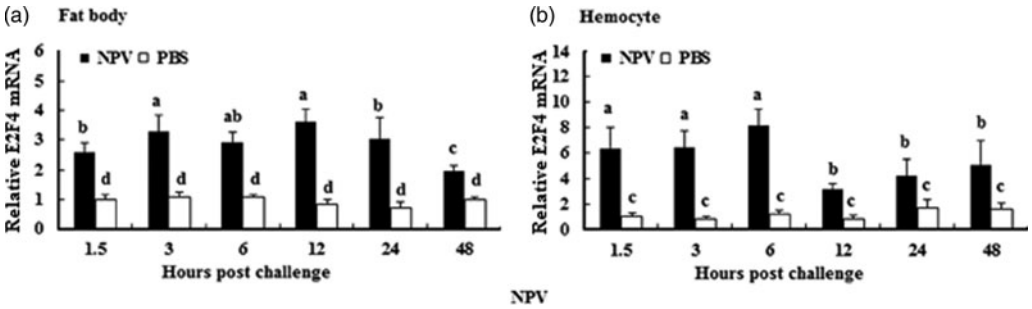

Fig. 5. Expression profiles of the E2F4 protein in haemocytes (a) and fat bodies (b) in the fifth-instar larval stage of *A. pernyi* from 1.5 to 48 h following nucleopolyhedrovirus challenge. The same letter indicates a non-significant difference, while values with different letters indicate a significant difference. The data were analysed using one-way analysis of variance. Data are represented as the means±standard error (SE). Differences were considered significant at $P < 0.05$.

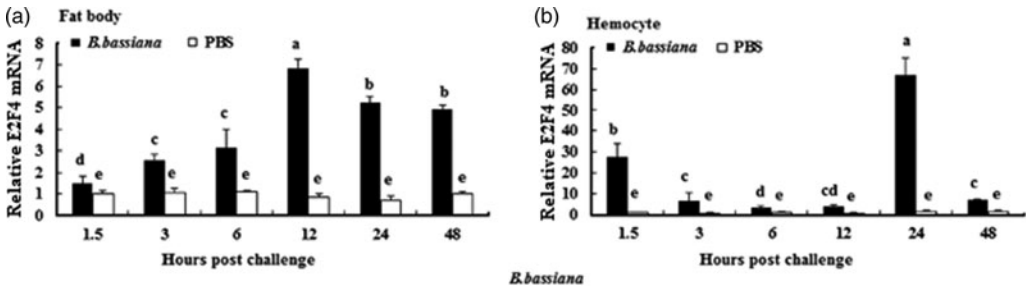

Fig. 6. Expression profiles of the E2F4 protein in (a) haemocytes and (b) fat bodies of *A. pernyi* fifth-instar larvae following *B. bassiana* challenge. The same letter indicates a non-significant difference while values with different superscript letters represent significant differences.

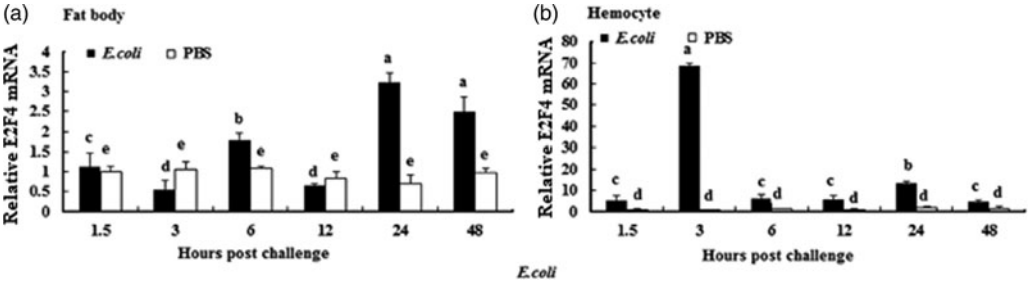

Fig. 7. Expression profiles of the E2F4 protein in (a) haemocytes and (b) fat bodies of *A. pernyi* fifth-instar larvae following *E. coli* challenge. The same letter indicates a non-significant difference while values with different superscript letters represent significant differences.

and then synthesized cDNA to determine the relative expression of mRNA of E2F4 at these time points. As seen in fig. 9, *E2F4* mRNA was expressed at high levels in the 1st instar larvae, while the lowest expression was recorded in the egg, and the 3rd and 5th instars.

Discussion and conclusions

In the present study, we amplified the *E2F4* cDNA from *A. pernyi*, and performed sequence analysis. The recombinant vector pET30a-E2F4 was constructed, which was transformed into *E. coli* and the recombinant protein was expressed successfully. Based on protein alignments and phylogenetic analysis of the deduced amino acid sequence, E2F4 is highly homologous to E2F4 proteins from other Lepidopterans, especially *Bombyx mori* (65%), but shows low homology to that from *Zootermopsis nevadensis* (53%), which suggested that that the *E2F4* genes are species specific.

E2F4 is involved mainly in the synthesis of DNA in undifferentiated cells (van Amerongen *et al.*, 2010; Ren *et al.*, 2012). However, recent studies revealed that E2F4 plays a variety of roles in differentiated cells, e.g. as a regulator of apoptosis and in tumour suppression, and as technology advances, further functions might be revealed (Zheng *et al.*, 1999; Cam &

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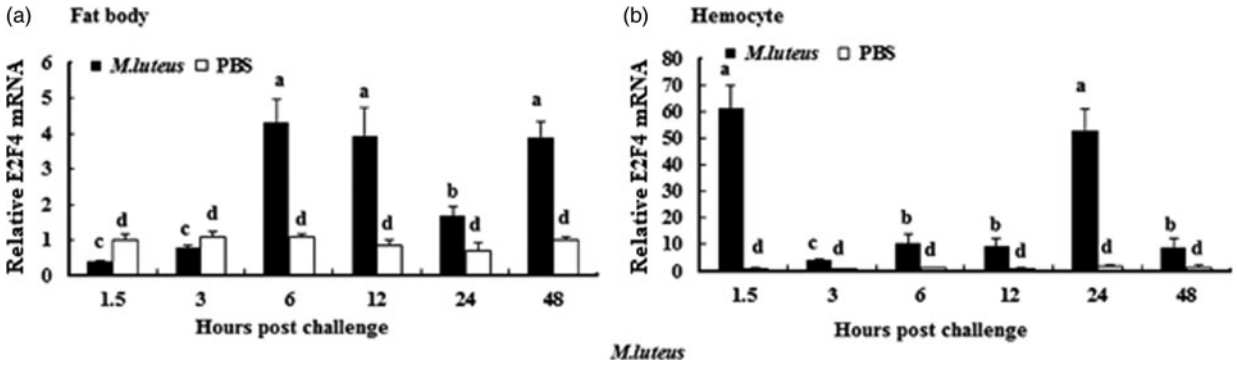

Fig. 8. Expression profiles of the E2F4 protein in (a) haemocytes and (b) fat bodies of *A. pernyi* fifth-instar larvae following *Micrococcus luteus* challenge. The same letter indicates a non-significant difference, while values with different superscript letters represent significant differences.

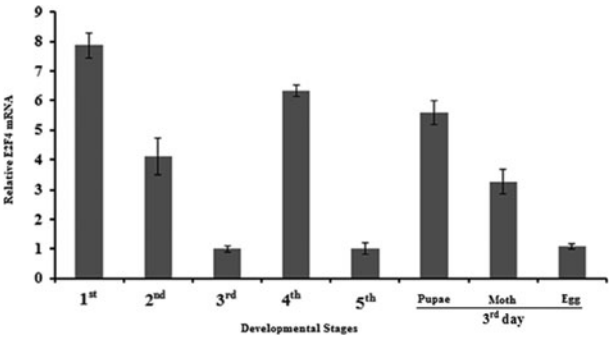

Fig. 9. Expression profiles of the E2F4 protein in different developmental stages of *A. pernyi*.

Dynlacht, 2003; Dingar *et al*., 2012). In the present study, we characterized E2F4 by evaluating its developmental expression profile, its expression in different tissues, and its response in immune tissue (fat body and haemocyte) to viral, fungal and bacterial challenge. Interestingly, E2F4 expression was high in the integument, fat body, and haemocytes compared with that in the other tissues examined. This indicated that the level of *E2F4* mRNA is tissue specific and might change depending on its function. Kusek *et al*. (2000) also documented variable patterns of *E2F4* mRNA expression in different tissues in both the embryonic and adult stages of mice.

To explore the role of E2F4 in response to microbial infection, the expression levels of E2F4 were determined after challenge by four types of pathogen antigens at different time intervals. The results revealed that, although the *E2F4* gene can be induced by all four pathogens, the maximum transcript level achieved in response to each pathogen appeared at different times. The response of the *E2F4* gene to the different pathogens in the fat bodies and haemocyte varied. In the fat bodies, NPV, *B. bassiana*, and *E. coli* treatment elicited high expression at 12 h, whereas *M. luteus* elicited high expression at 6 h after treatment. However in haemocytes, the expression of the transcript fluctuates largely with time and pathogen type. NPV induced maximum expression at 6 h, whereas *B. bassiana* induced it at 5 h. The transcript expression was much higher in response to *E. coli* and *M. luetus*; however, the maximum level was achieved at different times: at 3 h for *E. coli* and at 24 h for *M. luteus*. Our data suggested that *E2F4* can be induced by microbial infection in *A. pernyi*, and exhibited time-dependent increases during infection. However, the expression pattern of *E2F4* was different when challenged with the different pathogens. These observations were consistent with the study of Isomoto *et al*. (2002), who reported that the expression of E2F transcription factors is enhanced following biotic stress (*Helicobacter pylori* challenge) in mucosal cells. In addition, abiotic stress (Infra Red Radiation) can also increase the expression of E2F4 (DuPree *et al*., 2004). Furthermore, Bernales *et al*. (2008) observed differential expression of genes that are regulated by E2F, and suggested that an altered RB/E2F pathway could account for this differential expression of the target genes in primary anti-phospholipid syndrome. Further studies are required to determine the mechanism by which the expression of *E2F4* changes following microbial infection. Furthermore, whether the immune system is controlled by pathways related to E2F4 or E2F4 is controlled by pathways related to the immune system should be determined in future research.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485317000426

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